

**NON-PROVISIONAL PATENT APPLICATION**

**NEW TUMOR SUPPRESSOR GENE P33ING2**

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The Government of the United States of America as represented by the  
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### **CROSS-REFERENCES TO RELATED APPLICATIONS**

This application claims priority to provisional application U.S.S.N. 60/121,891, filed February 26, 1999, the disclosure of which is herein incorporated by reference in its entirety.

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### **STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT**

Not applicable.

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### **FIELD OF THE INVENTION**

This invention relates to isolated nucleic acid and amino acid sequences of novel human tumor suppressors, antibodies to such tumor suppressors, methods of detecting such nucleic acids and proteins, methods of screening for modulators of tumor suppressors, and methods of diagnosing and treating tumors with such nucleic acids and proteins.

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### **BACKGROUND OF THE INVENTION**

Certain tumors, benign, pre-malignant, and malignant, are known to have genetic components. Some of these tumors are caused by mutations or inactivation of "tumor suppressor" genes. In normal cells, the tumor suppressor genes are involved in the regulation of cell growth and proliferation and in the control of cellular aging, anchorage dependence and apoptosis. When the tumor suppressor genes are mutated or inactivated, cells are transformed and become immortalized or tumorigenic. These transformed cells can be reverted back to the normal phenotype (i.e., the cell growth rate is suppressed) by introducing the wildtype suppressor genes.

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The first tumor suppressor gene identified was the nuclear phosphoprotein, retinoblastoma gene (Rb). Retinoblastoma is a malignant tumor of the sensory layer of the retina, and often occurs bilaterally during childhood. Retinoblastoma exhibits a

familial tendency, but it can be acquired. Mutations in the Rb gene and inactivation of its product have been shown to be involved in other tumors, such as bladder, breast, small cell lung carcinomas, osteosarcomas, and soft tissue sarcomas. It was demonstrated that reconstitution of Rb-deficient tumor cells with the wildtype Rb leads to the suppression of growth rate or tumorigenicity (Huang *et al.*, *Science* 242:1563-1566 (1988)). This result provides direct evidence that Rb protein is a tumor suppressor.

Another well-characterized tumor suppressor is the gene for the nuclear phosphoprotein, p53. More than half of all human cancers are associated with mutations in the tumor suppressor gene p53 (*see, e.g.*, Hollstein *et al.*, *Science* 253:49-53 (1991); Caron de Fromental & Soussi, *Genes Chromosom. Cancer* 4: 1-15; Harris & Hollstein, *N. Engl. J. Med.* 329:1318-1327 (1993); Greenblatt *et al.*, *Cancer Res.* 54:4855-4878 (1994)). Mutations in p53 often appear to be a critical step in the pathogenesis and progression of tumors. For example, missense mutations of p53 occur in tumors of the colon, lung, breast, ovary, bladder, and several other organs. Alternatively, inactivation of the wildtype p53 proteins in cells can cause tumors. For example, certain strains of human papillomavirus (HPV) are known to interfere with the p53 protein function, because the virus produces a protein, E6, which promotes the degradation of the p53 protein.

Recently, another tumor suppressor gene, p33ING1, has been identified. p33ING1 directly cooperates with tumor suppressor gene p53 in growth regulation (Garkavtsev *et al.*, *Nature Genetics* 14:415-420 (1996); Garkavtsev *et al.*, *Nature* 391:295-298 (1998); U.S. Patent No. 5,986,078, all of which are herein incorporated by reference). Neither of p53 or p33ING1 can alone cause growth inhibition when the other one is suppressed (Garkavtsev *et al.* (1998), *supra*). According to immunoprecipitation studies, p33ING1 proteins modulate the p53 activity through physical interaction. It has been also reported that some neuroblastoma cells have a mutation of the p33ING1 gene, and some breast cancer cell lines exhibit reduced expression of p33ING1 (Garkavtsev *et al.* (1996), *supra*).

Cancer remains a major public concern. Although epidemiological and cytogenetic studies demonstrated that a number of recessive genetic mutations are involved in various cancers, only a limited number of tumor suppressors have been identified. Therefore, there is a need to identify and isolate other tumor suppressor genes. The identification and isolation of new tumor suppressor genes would assist the diagnosis, prevention, and treatment of tumors and cancers.

## SUMMARY OF THE INVENTION

The present invention thus provides for the first time nucleic acid and amino acid sequences of a new tumor suppressor gene called p33ING2, as well as antibodies to p33ING2, methods of detecting such nucleic acids and proteins, methods of screening for modulators of p33ING2, and methods of diagnosing and treating tumors. P33ING2 nucleic acids and proteins are tumor suppressors that play a key role in regulation of cell proliferation and tumor suppression.

In one aspect, the present invention provides an isolated nucleic acid encoding a tumor suppressor polypeptide p33ING2, wherein the polypeptide has greater than 70% amino acid sequence identity to a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In one embodiment, the nucleic acid encodes a polypeptide that selectively binds to polyclonal antibodies generated against a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In another embodiment, the nucleic acid encodes a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In yet another embodiment, the nucleic acid comprises a nucleotide sequence of SEQ ID NO:2. In yet another embodiment, the nucleic acid is from human. In yet another embodiment, the nucleic acid is amplified by primers that selectively hybridize under stringent hybridization conditions to the same sequence as degenerate primer sets encoding amino acid sequences selected from the group consisting of: SEQ ID NO:3 (MLGQQQQ) and SEQ ID NO:4 (KKDRRSR). In yet another embodiment, the nucleic acid encodes a polypeptide having a molecular weight of about 28 kDa to about 38 kDa. In yet another embodiment, the nucleic acid encodes a tumor suppressor polypeptide p33ING2 that specifically hybridizes under stringent conditions to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2. In yet another embodiment, the nucleic acid selectively hybridizes under moderately stringent hybridization conditions to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2.

In another aspect, the present invention provides an isolated tumor suppressor polypeptide p33ING2, wherein the polypeptide has greater than 70 % amino acid sequence identity to a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In one embodiment, the tumor suppressor polypeptide selectively binds to polyclonal antibodies generated against a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In another embodiment, the polypeptide is from human. In yet another embodiment, the polypeptide is wildtype p33ING2.

In yet another aspect, the present invention provides an antibody that selectively binds to a p33ING2 polypeptide comprising an amino acid sequence of SEQ ID NO:1, but does not bind to a p33ING1 polypeptide comprising an amino acid sequence of SEQ ID NO:8. In one embodiment, the antibody is polyclonal. In another embodiment, the antibody selectively binds to a p33ING2 polypeptide comprising the amino acid sequence of SEQ ID NO:5, but does not bind to a p33ING1 polypeptide comprising an amino acid sequence of SEQ ID NO:8.

In yet another aspect, the present invention provides an expression vector comprising any one or more of the p33ING2 nucleic acid described herein. The invention also provides a host cell transfected with a vector comprising any one or more of the p33ING2 nucleic acid described herein.

In yet another aspect, the present invention provides a method for identifying a compound that modulates a tumor suppressor polypeptide p33ING2, the method comprising the steps of: (i) contacting the compound with a eukaryotic host cell or cell membrane in which has been expressed a tumor suppressor polypeptide p33ING2, wherein the polypeptide has greater than 70 % amino acid sequence identity to a polypeptide comprising an amino acid sequence of SEQ ID NO:1; and (ii) determining the functional effect of the compound upon the cell or cell membrane expressing the polypeptide. In one embodiment of the method, the polypeptide selectively binds to polyclonal antibodies generated against a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In another embodiment of the method, functional effect is determined by measuring changes in cell growth. In yet another embodiment of the method, the polypeptide is recombinant. In yet another embodiment of the method, the polypeptide is from a human. In yet another embodiment of the method, the polypeptide comprises an amino acid sequence of SEQ ID NO:1. In yet another embodiment of the method, the cell is an HCT116 human colon cancer cell line. In yet another embodiment of the method, the cell has the missense p33ING2 sequence of a polypeptide comprising an amino acid sequence of SEQ ID NO:6.

In yet another aspect, the present invention provides a method of inhibiting cellular proliferation, the method comprising transducing a cell with an expression vector, the vector comprising a nucleic acid encoding a tumor suppressor polypeptide p33ING2, wherein the polypeptide has greater than 70 % amino acid sequence identity to a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In one embodiment of the method, the polypeptide selectively binds to polyclonal antibodies generated against a

polypeptide comprising an amino acid sequence of SEQ ID NO:1. In another embodiment of the method, the nucleic acid encodes a polypeptide comprising an amino acid sequence of SEQ ID NO:1. In yet another embodiment of the method, the nucleic acid comprises a nucleotide sequence of SEQ ID NO:2. In yet another embodiment of the method, the nucleic acid is from human. In yet another embodiment of the method, the nucleic acid encodes a polypeptide having a molecular weight of about 28 kDa to about 38 kDa. In yet another embodiment of the method, the cell has a missense or null endogenous p33ING2 phenotype. In yet another embodiment of the method, the cell has a missense p33ING2 sequence of a polypeptide comprising an amino acid sequence of SEQ ID NO:6.

In yet another aspect, the present invention provides a method of detecting the presence or absence of p33ING2 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a p33ING2-specific reagent that selectively associates with p33ING2; and (iii) detecting the level of p33ING2-specific reagent that selectively associates with the sample. In one embodiment of the method, the p33ING2-specific reagent is selected from the group consisting of a p33ING2-specific antibody, a p33ING2-specific primer, and a p33ING2-specific nucleic acid probe. In another embodiment of the method, the p33ING2-specific nucleic acid probe binds to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:7, or to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:2, or to a nucleic acid comprising a nucleotide sequence of SEQ ID NO:10. In yet another embodiment of the method, the biological sample comprises intact chromosome 4q35. In yet another embodiment of the method, the p33ING2-specific reagent detects nucleic acid, such as DNA or RNA. In yet another embodiment of the method, the nucleic acid is a polymorphic variant of p33ING2. In yet another embodiment of the method, the p33ING2-specific reagent is an antibody that selectively binds to p33ING2. In some embodiments, the antibody is polyclonal. In yet another embodiment of the method, the antibody selectively binds to a p33ING2 polypeptide comprising an amino acid sequence of SEQ ID NO:1, but not to a p33ING1 polypeptide comprising an amino acid sequence of SEQ ID NO:8. In yet another embodiment of the method, the antibody selectively binds to a p33ING2 polypeptide comprising an amino acid sequence of SEQ ID NO:5, but does not bind to a p33ING1 polypeptide comprising an amino acid sequence of SEQ ID NO:8.

In yet another aspect, the present invention provides a method of determining a test amount of p33ING2 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a p33ING2-specific reagent that selectively associates with p33ING2; and (iii) comparing the test amount to a control. In one embodiment, the control is an amount of p33ING2 in a normal cell. In another embodiment, the p33ING2-specific reagent is selected from the group consisting of p33ING2-specific antibody, a p33ING2-specific primer; and p33ING2-specific nucleic acid probe.

In yet another aspect, the present invention provides a method of detecting the presence or absence of p33ING1 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a p33ING1-specific antibody that selectively binds to p33ING1 but not to p33ING2; and (iii) detecting the level of p33ING1-specific antibody that selectively associates with the sample. In one embodiment, the p33ING1-specific antibody is polyclonal.

In yet another aspect, the present invention provides a method of determining a test amount of p33ING1 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a p33ING1-specific antibody that selectively associates with p33ING1 but not to p33ING2; and (iii) comparing the test amount to a control. In one embodiment, the control is an amount of p33ING1 in a normal cell. In another embodiment, the p33ING1-specific antibody is polyclonal.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates binding specificities of polyclonal antibodies for p33ING2 and polyclonal antibodies for p33ING1 by ELISA.

Figure 2 illustrates binding specificities of polyclonal antibodies for p33ING2 and polyclonal antibodies for p33ING1 by Western blot analysis.

Figure 3 illustrates that p33ING2 inhibits cell growth of HCT116 cell line by colony formation assay.

Figure 4 illustrates a Western blot that shows that p33ING2 protein is induced by topoisomerase II inhibitor, etoposide.

Figure 5 illustrates FACSscan flow cytometric data that shows that p33ING1 or p33ING2 can induce G<sub>1</sub> cell cycle arrest.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Introduction

The present invention provides for the first time nucleic acids and polypeptides of a new tumor suppressor called p33ING2. The present invention also provides antibodies which selectively bind to a p33ING2 protein, but not to a p33ING1 protein; and antibodies which selectively bind to a p33ING1 protein, but not to a p33ING2 protein. These nucleic acids and the polypeptides they encode are tumor suppressors. These tumor suppressor nucleic acids and polypeptides are involved in the regulation of cell proliferation and in the control of cellular aging, anchorage dependence, and apoptosis.

The present invention also provides methods of screening for modulators (e.g., activators, inhibitors, stimulators, enhancers, agonists, and antagonists) of these novel p33ING2 proteins. Such modulators are useful for pharmacological and genetic modulation of cell growth and tumor suppression. The invention thus provides assays for tumor suppression and cell growth, where p33ING2 acts as a direct or indirect reporter molecule for measuring the effect of modulators on cell growth or tumor suppression. These assays can measure various parameters that are affected by the p33ING2 activity, e.g., cell growth on soft agar, contact inhibition and density limitation of growth, growth factor or serum dependence, tumor specific markers levels, invasiveness into Matrigel, tumor growth *in vivo*, p33ING2 protein or mRNA levels, transcriptional activation or repression of a reporter gene, and the like.

The present invention also provides methods of inhibiting cell proliferation of a cell by transducing the cell with an expression vector containing p33ING2 nucleic acids. The transduced cell may have a missense or null endogenous p33ING2 phenotype or a mutation in another tumor suppressor gene. For example, the cell may contain p33ING2 having a sequence of SEQ ID NO:6 with a missense mutation. Expression of wildtype p33ING2 restores cell growth regulation and prevents the development of tumor. For example, p33ING2 nucleic acids can be used to treat cancer or other cell proliferative diseases, such as hyperplasia, in patients.

Finally, the invention provides for methods of detecting p33ING2 or p33ING1 nucleic acid and protein expression, allowing investigation of cell growth regulation and tumor suppression. Furthermore, p33ING2 or p33ING1 nucleic acid and protein expression can be used to diagnose cancer in patients who have a defect in one or more copies of p33ING2 or p33ING1 in their genome.



Functionally, p33ING2 represents a nuclear protein having a molecular weight of approximately 33 kDa. It is involved in the regulation of cell proliferation and in the control of cellular aging, anchorage and apoptosis.

Structurally, the nucleotide sequence of p33ING2 (*see, e.g.*, SEQ ID NO:2, isolated from a human) encodes a polypeptide of approximately 270 amino acids with a predicted molecular weight of approximately 33 kDa and a predicted range of 28-38 kDa (*see, e.g.*, SEQ ID NO:1). Related p33ING2 genes from other species share at least about 70% amino acid identity over an amino acid region of at least about 25 amino acids in length, preferably 50 to 100 amino acids in length.

Specific regions of the p33ING2 nucleotide and amino acid sequences may be used to identify polymorphic variants, interspecies homologs, and alleles of p33ING2. This identification can be made *in vitro*, e.g., under stringent hybridization conditions or with PCR and sequencing, or by using the sequence information in a computer system for comparison with other nucleotide or amino acid sequences. Typically, identification of polymorphic variants and alleles of p33ING2 is made by comparing an amino acid sequence of about 25 amino acids or more, preferably 50-100 amino acids. Amino acid identity of approximately at least 70% or above, preferably 80%, most preferably 90-95% or above typically demonstrates that a protein is a polymorphic variant, interspecies homolog, or allele of p33ING2. Sequence comparison can be performed using any of the sequence comparison algorithms discussed below. Antibodies that bind specifically to p33ING2 or a conserved region thereof can also be used to identify alleles, interspecies homologs, and polymorphic variants.

Polymorphic variants, interspecies homologs, and alleles of p33ING2 are confirmed by examining the effect of putative p33ING2 expression on cell growth and tumor suppression using the methods and assays described herein. Typically, p33ING2 having the amino acid sequence of SEQ ID NO:1 is used as a positive control. For example, immunoassays using antibodies directed against the amino acid sequence of SEQ ID NOS:1 or 5 can be used to demonstrate the identification of a polymorphic variant or allele of p33ING2. Alternatively, p33ING2 having the nucleic acid sequences of SEQ ID NO:1 is used as a positive control, e.g., in *in situ* hybridization with SEQ ID NO:1 to demonstrate the identification of a polymorphic variant or allele of p33ING2. The polymorphic variants, alleles and interspecies homologs of p33ING2 are expected to retain the ability to inhibit cell proliferation and tumor suppression. These functional characteristics can be tested using various assays, such as soft agar assay, contact

inhibition and density limitation of growth assay, growth factor or serum dependence assay, tumor specific markers assay, invasiveness assay, apoptosis assay, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest assay, tumor growth assay, etc.

5 The present invention also provides polymorphic variants of p33ING2 depicted in SEQ ID NO:1: variant #1, in which a threonine residue is substituted for a serine residue at amino acid position 11; variant #2, in which a leucine residue is substituted for an isoleucine residue at amino acid position 101; and variant #3, in which an alanine residue is substituted for a glycine residue at amino acid position 251.

10 P33ING2 nucleotide and amino acid sequence information may also be used to construct models of tumor suppressor polypeptides in a computer system. These models are subsequently used to identify compounds that can activate or inhibit p33ING2. Such compounds that modulate the activity of p33ING2 can be used to investigate the role of p33ING2 in inhibition of cell proliferation and tumor suppression or can be used as therapeutics.

15 Isolation of p33ING2 provides a means for assaying for modulators of p33ING2. P33ING2 is useful for testing modulators using *in vivo* and *in vitro* expression that measure various parameters, e.g., cell growth on soft agar, contact inhibition and density limitation of growth, growth factor or serum dependence, tumor specific markers levels, invasiveness into Matrigel, apoptosis assay, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest assay, tumor growth *in vivo*, p33ING2 protein or mRNA levels, transcriptional activation or repression of a reporter gene, and the like. Such modulators identified using p33ING2 can be used to study cell growth regulation and tumor suppression, and further to treat cancer.

20 Methods of detecting p33ING2 nucleic acids and expression of p33ING2 are also useful for diagnosing various cancers or tumors by using assays such as northern blotting, dot blotting, *in situ* hybridization, RNase protection, and the like. Chromosome localization of the genes encoding human p33ING2 can also be used to identify diseases, mutations, and traits caused by and associated with p33ING2. Techniques, such as high density oligonucleotide arrays (GeneChip<sup>TM</sup>), can be also be used to screen for mutations, polymorphic variants, alleles and interspecies homologs of p33ING2.

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## II. Definitions

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "tumor suppressor" refers to a gene, or the protein it encodes, that in its wildtype form has the ability to suppress, prevent, or decrease cell transformation. Tumor suppressor genes are genes that encode protein(s) that regulate cell growth and proliferation directly or indirectly, e.g., p53, Rb, and the like. If a tumor suppressor gene is damaged (e.g., by radiation, a carcinogen or inherited, or spontaneous mutation), it may lose its wildtype ability to regulate cell growth and proliferation, and the cells may become transformed or pre-disposed to transformation.

"p33ING" refers to a family of tumor suppressor nucleic acids or polypeptides having a molecular weight of approximately 33 kDa. They encode a nuclear protein which is involved in the regulation of cell growth and proliferation and in the control of cellular aging, anchorage and apoptosis. "p33ING2" and "p33ING1" are members of the "p33ING" family, which members are encoded by different genes (i.e., mapped to different regions on the chromosome). p33ING2 is mapped to human chromosome 4q35.

The term p33ING2 therefore refers to polymorphic variants, alleles, interspecies homologs, and mutants that: (1) have about 70% amino acid sequence identity, preferably about 80-90% amino acid sequence identity to SEQ ID NO:1 over a window of about at least 50-100 amino acids; (2) binds to polyclonal antibodies raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1 and conservatively modified variants thereof, but does not bind to polyclonal antibodies raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8 and conservatively modified variants thereof; (3) specifically hybridize (with a size of at least about 500, preferably at least about 900 nucleotides) under stringent hybridization conditions to a sequence selected from the group consisting of SEQ ID NO:2, and conservatively modified variants thereof; or (4) are amplified by primers that specifically hybridize under stringent conditions to the same sequence as a degenerate primers sets encoding SEQ ID NOS:3 and 4.

The term p33ING1 refers to polymorphic variants, alleles, interspecies homologs, and mutants that: (1) have about 70% amino acid sequence identity, preferably about 80-90% amino acid sequence identity to SEQ ID NO:8 over a window of about at least 50-100 amino acids; (2) binds to polyclonal antibodies raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NO:8 and conservatively modified variants thereof, but does not bind to polyclonal antibodies

raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1 and conservatively modified variants thereof.

The phrases “polymorphic variant” and “allele” refer to forms of p33ING2 that occur in a population (or among populations) and that maintain wildtype p33ING2 activity as measured using one of the assays described herein.

The term “mutant” of p33ING2 refers to those mutants which are experimentally made or those which are found in tumor or cancer cells. Mutants of p33ING2 can be due to, e.g., truncation, elongation, substitution of amino acids, deletion, insertion, or lack of expression (e.g., due to promoter or splice site mutations, etc.). A mutant has activity that differs from the activity of wildtype p33ING2 by at least about 20% as measured using an assay described herein. For example, a mutant of p33ING2 can have a null mutation which results in absence of normal gene product at the molecular level or an absence of function at the phenotypic level. Another example is a missense mutation of p33ING2, where a substitution of amino acid(s) results in a change in the activity of the protein.

The phrase “missense or null endogenous p33ING2 phenotype” of a cell therefore refers to p33ING2 has a missense or null mutation so that the cell has a phenotype (e.g., soft agar growth, contact inhibition and density limitation of growth, etc.) which differs from a cell having a wildtype p33ING2.

An “expression vector” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

The term “transfect” or “transduce” refers to any way of getting a nucleic acid across a cell membrane, including electroporation, biolistics, injection, plasmid transfection, lipofection, viral transduction, lipid-nucleic acid complexes, naked DNA, etc

A “host cell” is a naturally occurring cell or a transformed cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be cultured cells, explants, cells *in vivo*, and the like. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHO, HeLa, HCT116, RK0 cells, and the like.

“Biological sample” include, but are not limited to, tissue isolated from humans, mice, and rats. In some embodiments, a sample of biological tissue or fluid

contains nucleic acids or polypeptides of p33ING2 and/or p33ING1. Biological samples may also include sections of tissues such as frozen sections taken from histological purposes. A biological sample is typically obtained from a eukaryotic organism, such as insects, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mouse, cow, dog, guinea pig, or rabbit, and most preferably a primate such as chimpanzees or humans.

“Tumor cell” refers to precancerous, cancerous, and normal cells in a tumor.

“Cancer cells”, “transformed” cells or “transformation” in tissue culture, refers to spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic DNA, or uptake of exogenous DNA, it can also arise spontaneously or following exposure to a carcinogen, thereby mutating an endogenous gene. Transformation is associated with phenotypic changes, such as immortalization of cells, aberrant growth control, and/or malignancy (*see*, Freshney, *Culture of Animal Cells a Manual of Basic Technique* (3<sup>rd</sup> ed. 1994)).

The term “cell cycle” refers to the cyclic biochemical and structural events occurring during growth of cells. The cell cycle is divided into periods called: G<sub>0</sub>, Gap<sub>1</sub> (G<sub>1</sub>), DNA synthesis (S), GAP<sub>2</sub> (G<sub>2</sub>), and mitosis (M).

The phrase “functional effects” in the context of assays for testing compounds that modulate p33ING2 mediated tumor suppression includes the determination of any parameter that is indirectly or directly under the influence of the p33ING2 protein. Functional effects include, e.g., anchorage dependence, contact inhibition and density limitation of growth, growth factor or serum dependence, tumor specific markers levels, invasiveness, tumor growth, p33ING2 protein mRNA levels, apoptosis, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, and the like, *in vitro*, *in vivo*, and *ex vivo*.

By “determining the functional effect” is meant assays for a compound that increases or decreases a parameter that is directly or indirectly under the influence of p33ING2. Such functional effects can be measured by any means known to those skilled in the art, e.g., soft agar assay, contact inhibition and density limitation of growth assay, growth factor or serum dependence assay, tumor specific markers assay, invasiveness assay, apoptosis assay, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest assay, tumor growth assay, p33ING2 protein mRNA level assay, transcriptional activation or repression of a reporter gene assay, and the like, *in vitro*, *in vivo*, and *ex vivo*.



been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

A “promoter” is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription.

A “constitutive” promoter is a promoter that is active under most environmental and developmental conditions. An “inducible” promoter is a promoter that is active under environmental or developmental regulation.

The term “operably linked” refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term “heterologous” when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

A “label” is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include <sup>32</sup>P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the polypeptide of SEQ ID NO:1 can be made detectable, e.g., by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

The terms “isolated,” “purified,” or “biologically pure” refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated p33ING2 nucleic acid is separated from open reading frames that flank the p33ING2 gene and encode proteins other than p33ING2. The term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to



compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group. Examples of amino acid analogs include homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

“Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids which encode identical or essentially identical amino acid sequences. Where the nucleic does not encode an amino acid sequence (e.g., a ribosomal RNA), conservatively modified variants refer to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid.

Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Serine (S), Threonine (T);
- 3) Aspartic acid (D), Glutamic acid (E);
- 4) Asparagine (N), Glutamine (Q);
- 5) Cysteine (C), Methionine (M);
- 6) Arginine (R), Lysine (K), Histidine (H);
- 7) Isoleucine (I), Leucine (L), Valine (V); and
- 8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(see, e.g., Creighton, *Proteins* (1984)).

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 70% identity, preferably 75%, 80%, 85%, 90%, or 95% identity or higher over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to the complement of a test sequence. Preferably, the identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length. In most preferred embodiments, the sequences are substantially identical over the entire length of, e.g., the coding region.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison

algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment

score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always  $> 0$ ) and N (penalty score for mismatching residues; always  $< 0$ ). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of

sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux *et al.*, *Nuc. Acids Res.* 12:387-395 (1984)).

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

The phrase “selectively (or specifically) hybridizes to” refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase “stringent hybridization conditions” refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, “Overview of principles of hybridization and the strategy of nucleic acid assays” (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at

equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary “moderately stringent hybridization conditions” include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

“Antibody” refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

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Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'<sub>2</sub>, a dimer of Fab which itself is a light chain joined to V<sub>H</sub>-C<sub>H</sub>1 by a disulfide bond. The F(ab)'<sub>2</sub> may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'<sub>2</sub> dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (*see, e.g., McCafferty et al., Nature* 348:552-554 (1990)).

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g., Kohler & Milstein, Nature* 256:495-497 (1975); Kozbor *et al., Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g., McCafferty et al., Nature* 348, 552-554 (1990); Marks *et al., Biotechnology* 10, 779-783 (1992)).

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, *etc.*; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

An "anti-p33ING2" antibody is an antibody or antibody fragment that specifically binds a polypeptide encoded by the p33ING2 gene, cDNA, or a subsequence thereof.

An “anti-p33ING1” antibody is an antibody or antibody fragment that specifically binds to a polypeptide encoded by the p33ING1 gene, cDNA, or a subsequence thereof.

5 The term “immunoassay” is an assay that uses an antibody to specifically bind an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The phrase “specifically (or selectively) binds” to an antibody or “specifically (or selectively) immunoreactive with,” when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in  
10 a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to p33ING2 at least two times the background, more typically 10 to 100 times background, and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to a polyclonal antibody under such conditions may require an antibody that is selected for its  
15 specificity for a particular protein. For example, polyclonal antibodies raised to p33ING2 from a species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with p33ING2 and not with other proteins, such as p33ING1, except for polymorphic variants and alleles of p33ING2. This selection may be achieved for polyclonal antibodies by subtracting out  
20 antibodies that cross react with p33ING1. For monoclonal antibodies, the specificity may be achieved by using a p33ING2 specific antigen to make the hybridomas (*e.g.*, SEQ ID NO:5). *See, e.g.*, Figure 2. Similarly, polyclonal antibodies raised to p33ING1 from a species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with p33ING1 and not with other  
25 proteins, such as p33ING2, except for polymorphic variants and alleles of p33ING1 using the methods described above. For identifying p33ING2 or p33ING1 variants and alleles from a particular species such as a human, the selection may be achieved by subtracting out antibodies that cross-react with p33ING2 or p33ING1 molecules, respectively, from other species. For species specific monoclonal antibodies, a species specific antigen can  
30 be used to make the hybridomas. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory*



Manual (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

The phrase “selectively associates with” refers to the ability of a nucleic acid to “selectively hybridize” with another as defined above, or the ability of an antibody to “selectively (or specifically) bind to a protein, as defined above.

“p33ING2-specific reagent” refers to any reagent which specifically associates with p33ING2. For example, it can be a p33ING2-specific antibody, a p33ING2-specific primer, or a p33ING2-specific nucleic acid probe.

### III. Isolation of the gene encoding p33ING2

#### A. General recombinant DNA methods

P33ING2 polypeptides and nucleic acids are used in the assays described below. For example, recombinant p33ING2 can be used to make cells that constitutively express p33ING2. Such polypeptides and nucleic acids can be made using routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2<sup>nd</sup> ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

Oligonucleotides can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is typically by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983). The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, e.g., the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16:21-26 (1981). Again, as noted above, companies such as Operon

Technologies, Inc. provide an inexpensive commercial source for essentially any oligonucleotide.

*B. Cloning Methods for the isolation of nucleotide sequences encoding*

5 *p33ING2*

In general, the nucleic acid sequences encoding genes of interest, such as p33ING2 and related nucleic acid sequence homologs, are cloned from cDNA and genomic DNA libraries by hybridization with a probe, or isolated using amplification techniques with oligonucleotide primers. Preferably mammalian, more preferably human sequences are used. For example, p33ING2 sequences are typically isolated from mammalian nucleic acid (genomic or cDNA) libraries by hybridizing with a nucleic acid probe, the sequence of which can be derived from SEQ ID NO:1. A suitable tissue from which human p33ING2 RNA and cDNA can be isolated is, e.g., placenta, HepG2 or Saos-2 cell lines.

15 Amplification techniques using primers can also be used to amplify and isolate, e.g., a nucleic acid encoding p33ING2, from DNA or RNA (*see, e.g., Dieffenbach & Dveksler, PCR Primer: A Laboratory Manual* (1995)). These primers can be used, e.g., to amplify either the full length sequence or a probe of one to several hundred nucleotides, which is then used to screen a mammalian library for the full-length nucleic acid of choice. For example, degenerate primer sets, such as MLGQQQQ (SEQ ID NO:3) and KKDRRSR (SEQ ID NO:4) can be used to isolate p33ING2 nucleic acids. Nucleic acids can also be isolated from expression libraries using antibodies as probes. Such polyclonal or monoclonal antibodies can be raised, e.g., using the sequence of p33ING2.

25 Polymorphic variants and alleles that are substantially identical to the gene of choice can be isolated using nucleic acid probes, and oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone, e.g., p33ING2 and p33ING2 polymorphic variants, interspecies homologs, and alleles, by detecting expressed homologs immunologically with antisera or purified antibodies made against p33ING2, which also recognize and selectively bind to the p33ING2 homolog.

30 To make a cDNA library, one should choose a source that is rich in the mRNA of choice, e.g., for human p33ING2 mRNA, placenta, HepG2 or Saos-2 cell lines. The mRNA is then made into cDNA using reverse transcriptase, ligated into a

recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g., Gubler & Hoffman, Gene 25:263-269 (1983); Sambrook et al., supra; Ausubel et al., supra*).

5 For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in non-lambda expression vectors. These vectors are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton &  
10 Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al., Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating a nucleic acid and its homologs combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (*see U.S. Patents 4,683,195 and 4,683,202; PCR Protocols: A Guide to*  
15 *Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of, e.g., p33ING2 directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify p33ING2 homologs using the sequences provided herein. Restriction endonuclease sites can be  
20 incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of p33ING2 encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose  
25 gels and cloned into an appropriate vector.

As described above, gene expression of p33ING2 or p33ING1 can also be analyzed by techniques known in the art, e.g., reverse transcription and PCR amplification of mRNA, isolation of total RNA or poly A<sup>+</sup> RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, probing high density oligonucleotides,  
30 and the like. All of these techniques are standard in the art.

Synthetic oligonucleotides can be used to construct recombinant genes for use as probes or for expression of protein. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and non-sense strands of the gene. These DNA fragments are then annealed, ligated and

cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of the p33ING2 nucleic acid. The specific subsequence is then ligated into an expression vector.

The nucleic acid encoding the protein of choice is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors. Optionally, cells can be transfected with recombinant p33ING2 operably linked to a constitutive promoter, to provide higher levels of p33ING2 expression in cultured cells.

### *C. Expression in prokaryotes and eukaryotes*

To obtain high level expression of a cloned gene or nucleic acid, such as those cDNAs encoding p33ING2, one typically subclones p33ING2 into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the p33ING2 protein are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function. The promoter typically can also include elements that are responsive to transactivation, e.g., hypoxia responsive elements, Gal4 responsive elements, lac repressor responsive elements, and the like. The promoter can be constitutive or inducible, heterologous or homologous.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked, e.g., to the nucleic acid sequence encoding p33ING2, and signals required for efficient polyadenylation of the transcript, ribosome

binding sites, and translation termination. The nucleic acid sequence may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical (one expression vector is described in Example I). Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a p33ING2 encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit

selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of protein, which are then purified using standard techniques (*see, e.g., Colley et al., J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification, in Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g., Morrison, J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g., Sambrook et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of the p33ING2 protein, which is recovered from the culture using standard techniques identified below.

#### IV. Purification of p33ING2

If necessary, naturally occurring or recombinant proteins can be purified for use in functional assays, e.g., to make antibodies to detect p33ING2. Naturally occurring p33ING2 is purified, e.g., from mammalian tissue such as placenta, HepG2 or Saos-2 cell lines or any other source of a p33ING2 homolog. Recombinant p33ING2 is purified from any suitable expression system, e.g., by expressing p33ING2 in *E. coli* and then purifying the recombinant protein via affinity purification, e.g., by using antibodies that recognize a specific epitope on the protein or on part of the fusion protein, or by

using glutathione affinity gel, which binds to GST. In some embodiments, the recombinant protein is a fusion protein, e.g., with GST or Gal4 at the N-terminus.

The protein of choice may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.,* Scopes, 5 *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook *et al., supra*).

A number of procedures can be employed when recombinant protein is being purified. For example, proteins having established molecular adhesion properties 10 can be reversibly fused to p33ING2. With the appropriate ligand, p33ING2 can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, p33ING2 could be purified using immunoaffinity columns.

#### 15 *A. Purification of p33ING2 from recombinant bacteria*

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for 20 isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates (“inclusion bodies”). Several protocols are suitable for purification of inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation 25 in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g.,* Sambrook *et al., supra*; Ausubel *et al., supra*).

30 If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride

(from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity.

- 5 Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. The protein of choice is separated from other bacterial proteins by standard separation techniques, e.g.,  
10 with Ni-NTA agarose resin.

Alternatively, it is possible to purify the recombinant p33ING2 protein from bacteria periplasm. After lysis of the bacteria, when the protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate  
15 recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO<sub>4</sub> and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant  
20 can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

#### *B. Standard protein separation techniques for purifying p33ING2*

##### Solubility fractionation

- 25 Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate  
30 on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest



is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

#### Size differential filtration

The molecular weight of the protein, e.g., p33ING2, can be used to isolate the protein from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

#### Column chromatography

The protein of choice can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

### **V. Immunological detection of p33ING2 and p33ING1**

In addition to the detection of p33ING2 genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect p33ING2, e.g., to identify alleles, mutants, polymorphic variants and interspecies homologs of p33ING2. Immunoassays can be used to qualitatively or quantitatively analyze p33ING2, e.g., to detect p33ING2, to measure p33ING2 activity, or to identify modulators of p33ING2 activity. Similarly, immunoassay can be used to detect and analyze p33ING1. A general overview of the applicable technology can be found in Harlow and Lane, *Antibodies: A Laboratory Manual* (1988).

#### A. Antibodies to p33ING2 and p33ING1

Methods of producing polyclonal and monoclonal antibodies that react specifically with p33ING2 or p33ING1 are known to those of skill in the art (*see, e.g.,* Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2<sup>nd</sup> ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975)). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.,* Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)). In addition, as noted above, many companies, such as BMA Biomedicals, Ltd., HTI Bio-products, and the like, provide the commercial service of making an antibody to essentially any peptide.

A number of p33ING2 or p33ING1 comprising immunogens may be used to produce antibodies specifically reactive with p33ING2 or p33ING1, respectively. For example, recombinant p33ING2 or p33ING1, or antigenic fragments thereof, are isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used as an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. To improve reproducibility, an inbred strain of mice (e.g., BALB/C mice) can be immunized to make the antibody; however, standard animals (mice, rabbits, etc.) used to make antibodies are immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol (*see* Harlow & Lane, *supra*). The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the protein of choice. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see* Harlow & Lane, *supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see Kohler & Milstein, Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.*, *Science* 246:1275-1281 (1989).

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of  $10^4$  or greater are selected and tested for their cross reactivity against non-p33ING2 proteins or even other related proteins, e.g., from other organisms, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with  $K_D$  of at least about 0.1 mM, more usually at least about 1  $\mu$ M, preferably at least about 0.1  $\mu$ M or better, and most preferably, 0.01  $\mu$ M or better.

Once p33ING2 or p33ING1 specific antibodies are available, these proteins can be detected by a variety of immunoassay methods. For a review of immunological and immunoassay procedures, *see Basic and Clinical Immunology* (Stites & Terr eds., 7<sup>th</sup> ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme Immunoassay* (Maggio, ed., 1980); and Harlow & Lane, *supra*.

#### *B. Immunological binding assays*

P33ING2 or p33ING1 can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, *see also Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7<sup>th</sup> ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that

specifically binds to a protein or antigen of choice (in this case p33ING2, p33ING1, or antigenic fragments thereof). The antibody may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may itself be one of the moieties comprising the antibody/antigen complex. Thus, the labeling agent may be a labeled p33ING2 or p33ING1 polypeptide or a labeled anti-p33ING2 or anti-p33ING1 antibody. Alternatively, the labeling agent may be a third moiety, such a secondary antibody, that specifically binds to the antibody/antigen complex (a secondary antibody is typically specific to antibodies of the species from which the first antibody is derived). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, e.g., Kronval et al., J. Immunol.* 111:1401-1406 (1973); Akerstrom *et al., J. Immunol.* 135:2589-2542 (1985)). The labeling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

#### Non-competitive assay formats

Immunoassays for detecting p33ING2 or p33ING1 in samples may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In one preferred "sandwich" assay, for example, the anti-antigen antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture antigen present in the test sample. Antigen thus immobilized is then bound by a labeling agent, such as a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species



to compete with itself. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with the added proteins are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the added proteins.

Furthermore, immunoassays in the competitive binding format can be used to determine cross-reactivity of polyclonal anti-p33ING2 antibodies or p33ING1 antibodies for p33ING1 and p33ING2 proteins, respectively. As described above, p33ING2 protein can be immobilized to a solid support. p33ING1 protein is added to the assay, and the ability of p33ING1 protein to compete for binding of the antisera to the immobilized p33ING1 protein is compared to the ability of p33ING2 to compete with itself. Those antisera with less than 10% crossreactivity with p33ING1 protein are selected and pooled. Such immunoassays provides antibodies that selectively bind to a p33ING2 polypeptide but do not bind to a p33ING1 polypeptide. Similarly, immunoassays in the competitive binding format can be used to select antibodies that selectively bind to a p33ING1 polypeptide, but do not bind to a p33ING2 polypeptide. See, e.g., Figure 2.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein thought to be perhaps an allele, interspecies homologs, or polymorphic variant of p33ING2 or p33ING1, to the immunogen protein. In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required to inhibit 50% of binding is less than 10 times the amount of the first protein that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to the immunogen of choice.

#### Other assay formats

Western blot (immunoblot) analysis is used to detect and quantify the presence of p33ING2 or p33ING1 in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind p33ING2. The anti-antigen antibodies specifically bind

to the antigen on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled sheep anti-mouse antibodies) that specifically bind to the anti-antigen antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (e.g., antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)).

#### Reduction of non-specific binding

One of skill in the art will appreciate that it is often desirable to minimize non-specific binding in immunoassays. Particularly where the assay involves an antigen or antibody immobilized on a solid substrate, it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this technique involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

#### Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS™), fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to another molecule (e.g., streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize a specific protein, or secondary antibodies that recognize antibodies to the specific protein.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies.



In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

## 5 VI. Assays for measuring changes in p33ING2 regulated cell growth

P33ING2 and its alleles, interspecies homologs, and polymorphic variants participate in regulation of cell proliferation and tumor suppression. Therefore, expression of p33ING2 and its alleles, interspecies homologs, and polymorphic variants in host cells would inhibit cell proliferation and suppress tumor formation. On the other  
10 hand, expression of p33ING2 mutants in a cell could lead to abnormal cell proliferation and loss of tumor suppressor phenotypes. Finally, compounds that activate or inhibit p33ING2 would indirectly affect regulation of cellular proliferation and tumor suppression. Any of these changes in cell growth can be assessed by using a variety of *in vitro* and *in vivo* assays, e.g., ability to grow on soft agar, changes in contact inhibition  
15 and density limitation of growth, changes in growth factor or serum dependence, changes in the level of tumor specific markers, changes in invasiveness into Matrigel, changes in apoptosis, changes in cell cycle pattern, changes in tumor growth *in vivo*, such as in transgenic mice, etc. Furthermore, these assays can be used to screen for activators, inhibitors, and modulators of p33ING2. Such activators, inhibitors, and modulators of  
20 p33ING2 can then be used to modulate p33ING2 expression in tumor cells or abnormal proliferative cells.

### *A. Assays for changes in cell growth by expression of p33ING2 constructs*

One or more of the following assays can be used to identify p33ING2  
25 constructs which are capable of regulating cell proliferation and tumor suppression. The phrase “p33ING2 constructs” can refer to any of p33ING2 and its alleles, interspecies homologs, polymorphic variants and mutants. Functional p33ING2 constructs identified by the following assays can then be used in, e.g., gene therapy to inhibit abnormal cellular proliferation and transformation.

30

#### Soft agar growth or colony formation in suspension

Normal cells require a solid substrate to attach and grow. When the cells are transformed, they lose this phenotype and grow detached from the substrate. For example, transformed cells can grow in stirred suspension culture or suspended in semi-

solid media, such as semi-solid or soft agar. The transformed cells, when transfected with tumor suppressor genes, regenerate normal phenotype and require a solid substrate to attach and grow.

Soft agar growth or colony formation in suspension assays can be used to identify p33ING2 constructs, which when expressed in host cells, inhibit abnormal cellular proliferation and transformation. Typically, transformed host cells (e.g., cells that grow on soft agar) are used in this assay. For example, RKO or HCT116 cell lines can be used. Expression of a tumor suppressor gene in these transformed host cells would reduce or eliminate the host cells' ability to grow in stirred suspension culture or suspended in semi-solid media, such as semi-solid or soft. This is because the host cells would regenerate anchorage dependence of normal cells, and therefore require a solid substrate to grow. Therefore, this assay can be used to identify p33ING2 constructs that encode a functional tumor suppressor. Once identified, such p33ING2 constructs can be used in a number of diagnostic or therapeutic methods, e.g., in gene therapy to inhibit abnormal cellular proliferation and transformation.

Techniques for soft agar growth or colony formation in suspension assays are described in Freshney, *Culture of Animal Cells a Manual of Basic Technique*, 3<sup>rd</sup> ed., Wiley-Liss, New York (1994), herein incorporated by reference. *See also*, the methods section of Garkavtsev *et al.* (1996), *supra*, herein incorporated by reference.

#### Contact inhibition and density limitation of growth

Normal cells typically grow in a flat and organized pattern in a petri dish until they touch other cells. When the cells touch one another, they are contact inhibited and stop growing. When cells are transformed, however, the cells are not contact inhibited and continue to grow to high densities in disorganized foci. Thus, the transformed cells grow to a higher saturation density than normal cells. This can be detected morphologically by the formation of a disoriented monolayer of cells or rounded cells in foci within the regular pattern of normal surrounding cells. Alternatively, labeling index with [<sup>3</sup>H]-thymidine at saturation density can be used to measure density limitation of growth. *See* Freshney (1994), *supra*. The transformed cells, when transfected with tumor suppressor genes, regenerate a normal phenotype and become contact inhibited and would grow to a lower density.

Contact inhibition and density limitation of growth assays can be used to identify p33ING2 constructs which are capable of inhibiting abnormal proliferation and

transformation in host cells. Typically, transformed host cells (e.g., cells that are not contact inhibited) are used in this assay. For example, RKO or HCT116 cell lines can be used. Expression of a tumor suppressor gene in these transformed host cells would result in cells which are contact inhibited and grow to a lower saturation density than the transformed cells. Therefore, this assay can be used to identify p33ING2 constructs which function as a tumor suppressor. Once identified, such p33ING2 constructs can be used, e.g., in gene therapy to inhibit abnormal cellular proliferation and transformation.

In this assay, labeling index with [<sup>3</sup>H]-thymidine at saturation density is a preferred method of measuring density limitation of growth. Transformed host cells are transfected with a p33ING2 construct and are grown for 24 hours at saturation density in non-limiting medium conditions. The percentage of cells labeling with [<sup>3</sup>H]-thymidine is determined autoradiographically. *See, Freshney (1994), supra.* The host cells expressing a functional p33ING2 construct would give rise to a lower labeling index compared to control (e.g., transformed host cells transfected with a vector lacking an insert).

#### Growth factor or serum dependence

Growth factor or serum dependence can be used as an assay to identify functional p33ING2 constructs. Transformed cells have a lower serum dependence than their normal counterparts (*see, e.g., Temin, J. Natl. Cancer Insti. 37:167-175 (1966); Eagle et al., J. Exp. Med. 131:836-879 (1970); Freshney, supra.* This is in part due to release of various growth factors by the transformed cells. When a tumor suppressor gene is transfected and expressed in these transformed cells, the cells would reacquire serum dependence and would release growth factors at a lower level. Therefore, this assay can be used to identify p33ING2 constructs which encode functional tumor suppressor. Growth factor or serum dependence of transformed host cells which are transfected with a p33ING2 construct can be compared with that of control (e.g., transformed host cells which are transfected with a vector without insert). Host cells expressing a functional p33ING2 would exhibit an increase in growth factor and serum dependence compared to control.

#### Tumor specific markers levels

Tumor cells release an increased amount of certain factors (hereinafter "tumor specific markers") than their normal counterparts. For example, plasminogen activator (PA) is released from human glioma at a higher level than from normal brain

cells (see, e.g., Gullino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth*. In Mihich (ed.): "Biological Responses in Cancer." New York, Academic Press, pp. 178-184 (1985)). Similarly, Tumor angiogenesis factor (TAF) is released at a higher level in tumor cells than their normal counterparts. See, e.g.,  
5 Folkman, *Angiogenesis and cancer, Sem Cancer Biol.* (1992)).

Tumor specific markers can be assayed for to identify p33ING2 constructs, which when expressed, decrease the level of release of these markers from host cells. Typically, transformed or tumorigenic host cells are used. Expression of a tumor suppressor gene in these host cells would reduce or eliminate the release of tumor  
10 specific markers from these cells. Therefore, this assay can be used to identify p33ING2 constructs that encode a functional tumor suppressor.

Various techniques which measure the release of these factors are described in Freshney (1994), *supra*. Also, see, Unkless *et al.*, *J. Biol. Chem.* 249:4295-4305 (1974); Strickland & Beers, *J. Biol. Chem.* 251:5694-5702 (1976); Whur *et al.*, *Br.*  
15 *J. Cancer* 42:305-312 (1980); Gulino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth*. In Mihich, E. (ed): "Biological Responses in Cancer." New York, Plenum (1985); Freshney *Anticancer Res.* 5:111-130 (1985).

#### Invasiveness into Matrigel

20 The degree of invasiveness into Matrigel or some other extracellular matrix constituent can be used as an assay to identify p33ING2 constructs which are capable of inhibiting abnormal cell proliferation and tumor growth. Tumor cells exhibit a good correlation between malignancy and invasiveness of cells into Matrigel or some other extracellular matrix constituent. In this assay, tumorigenic cells are typically used  
25 as host cells. Expression of a tumor suppressor gene in these host cells would decrease invasiveness of the host cells. Therefore, functional p33ING2 constructs can be identified by measuring changes in the level of invasiveness between the host cells before and after the introduction of p33ING2 constructs. If a p33ING2 construct functions as a tumor suppressor, its expression in tumorigenic host cells would decrease invasiveness.

30 Techniques described in Freshney (1994), *supra*, can be used. Briefly, the level of invasion of host cells can be measured by using filters coated with Matrigel or some other extracellular matrix constituent. Penetration into the gel, or through to the distal side of the filter, is rated as invasiveness, and rated histologically by number of cells and distance moved, or by prelabeling the cells with <sup>125</sup>I and counting the

radioactivity on the distal side of the filter or bottom of the dish. *See, e.g., Freshney (1984), supra.*

#### Apoptosis analysis

5 Apoptosis analysis can be used as an assay to identify functional p33ING2 constructs. p33ING2 expression or overexpression causes apoptosis (*see* Example IX below). In this assay, cell lines, such as RKO or HCT116, can be used to screen p33ING2 constructs which encode a functional tumor suppressor. Cells are transfected with a putative p33ING2 construct. The cells can be co-transfected with a construct  
10 comprising a marker gene, such as a gene that encodes green fluorescent protein. Alternatively, a single construct comprising a putative p33ING2 gene and a marker gene can be transfected into cells. Overexpression of a p33ING2 gene that encodes a functional tumor suppressor would cause apoptosis. Not wishing to be bound by a theory, exogenous expression of a tumor suppressor can decrease cell proliferation by causing a  
15 cell cycle arrest and by increasing cell death. The apoptotic change can be determined using methods known in the art, such as DAPI staining and TUNEL assay using fluorescent microscope. For TUNEL assay, commercially available kit can be used (e.g., Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, Cat.# QIA39) + Tetramethyl-rhodamine-5-dUTP (Roche, Cat. # 1534 378)). Cells  
20 expressing a functional p33ING2 would exhibit an increased apoptosis compared to control (e.g., a cell transfected with a vector without a p33ING2 gene insert).

#### G<sub>0</sub>/G<sub>1</sub> cell cycle arrest analysis

25 G<sub>0</sub>/G<sub>1</sub> cell cycle arrest can be used as an assay to identify functional p33ING2 constructs. p33ING2 expression or overexpression causes G<sub>1</sub> cell cycle arrest (*see* Example IX below). In this assay, cell lines, such as RKO or HCT116, can be used to screen p33ING2 constructs which encode a functional tumor suppressor. Cells are transfected with a putative p33ING2 construct. The cells can be co-transfected with a construct comprising a marker gene, such as a gene that encodes green fluorescent  
30 protein. Alternatively, a single construct comprising a putative p33ING2 gene and a marker gene can be transfected into cells. Expression or overexpression of a p33ING2 gene that encodes a functional tumor suppressor would cause G<sub>0</sub>/G<sub>1</sub> cell cycle arrest (*see, e.g., Example VII*). Methods known in the art can be used to measure the degree of G<sub>1</sub> cell cycle arrest. For example, the propidium iodide signal can be used as a measure for

DNA content to determine cell cycle profiles on a flow cytometer. The percent of the cells in each cell cycle can be calculated. Cells expressing a functional p33ING2 would exhibit a higher number of cells that are arrested in G<sub>0</sub>/G<sub>1</sub> phase compared to control (e.g., transfected with a vector without a p33ING2 gene insert).

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#### Tumor growth *in vivo*

Effects of p33ING2 on cell growth can be tested in transgenic or immune-suppressed mice. Knock-out transgenic mice can be made, in which the endogenous p33ING2 gene is disrupted. Such knock-out mice can be used to study effects of p33ING2, e.g., as a cancer model, as a means of assaying *in vivo* for compounds that modulate p33ING2, and to test the effects of restoring a wildtype p33ING2 to a knock-out mice.

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Knock-out transgenic mice can be made by insertion of a marker gene or other heterologous gene into the endogenous p33ING2 gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting the endogenous p33ING2 with a mutated version of p33ING2, or by mutating the endogenous p33ING2, e.g., by exposure to carcinogens.

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A DNA construct is introduced into the nuclei of embryonic stem cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (*see, e.g., Capecchi et al., Science* 244:1288 (1989)). Chimeric targeted mice can be derived according to Hogan *et al., Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., IRL Press, Washington, D.C., (1987).

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These knock-out mice can be used as hosts to test the effects of various p33ING2 constructs on cell growth. These transgenic mice with the endogenous p33ING2 gene knocked out would develop abnormal cell proliferation and tumor growth. They can be used as hosts to test the effects of various p33ING2 constructs on cell growth. For example, introduction of wildtype p33ING2 into these knock-out mice would inhibit abnormal cellular proliferation and suppress tumor growth.

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Alternatively, various immune-suppressed or immune-deficient host animals can be used. For example, genetically athymic “nude” mouse (*see, e.g., Giovanella et al., J. Natl. Cancer Inst.* 52:921 (1974)), a SCID mouse, a thymectomized mouse, or an irradiated mouse (*see, e.g., Bradley et al., Br. J. Cancer* 38:263 (1978); Selby *et al., Br. J. Cancer* 41:52 (1980)) can be used as a host. Transplantable tumor cells (typically about  $10^6$  cells) injected into isogenic hosts will produce invasive tumors in a high proportions of cases, while normal cells of similar origin will not. In hosts which developed invasive tumors, cells expressing a p33ING2 construct are injected subcutaneously. After a suitable length of time, preferably 4-8 weeks, tumor growth is measured (e.g., by volume or by its two largest dimensions) and compared to the control. Tumors that have statistically significant reduction (using, e.g., Student’s T test) are said to have inhibited growth. Using reduction of tumor size as an assay, functional p33ING2 constructs which are capable of inhibiting abnormal cell proliferation can be identified. This model can also be used to identify mutant versions of p33ING2.

#### B. Assays for compounds that modulate p33ING2

P33ING2 and its alleles, interspecies homologs, and polymorphic variants participate in regulation of cell proliferation and tumor suppression. Mutations in these genes, including null or missense mutations, can cause abnormal cell proliferation and tumor growth. The activity of p33ING2 polypeptides (wildtype or mutants) can be assessed using a variety of *in vitro* and *in vivo* assays measuring various parameters, e.g., cell growth on soft agar, contact inhibition and density limitation of growth, growth factor or serum dependence, tumor specific markers levels, invasiveness into Matrigel, tumor growth *in vivo*, transgenic mice, p33ING2 protein or mRNA levels, transcriptional activation or repression of a reporter gene, apoptosis analysis, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, and the like. Such assays can also be used to screen for activators, inhibitors, and modulators of wildtype and mutant p33ING2. Such activators, inhibitors, and modulators are useful in inhibiting tumor growth and modulating cell proliferation. Compounds identified using the assays of the invention are useful as therapeutics for treatment of cancer and other diseases involving cellular hyperproliferation.

Biologically active or inactivated p33ING2 polypeptides, either recombinants or naturally occurring, are used to screen activators, inhibitors, or modulators of tumor suppression and cell proliferation. The p33ING2 polypeptides can be recombinantly expressed in a cell, naturally expressed in a cell, recombinantly or

naturally expressed in cells transplanted into an animal, or recombinantly or naturally expressed in a transgenic animal. Modulation is tested using one of the *in vitro* or *in vivo* assays described in herein in part A.

Cells that have wildtype p33ING2, p33ING2 null mutations, p33ING2 missense mutations, or inactivation of p33ING2 are used in the assays of the invention, both *in vitro* and *in vivo*. Preferably, human cells are used. Cell lines can also be created or isolated from tumors that have mutant p33ING2. Optionally, the cells can be transfected with an exogenous p33ING2 gene operably linked to a constitutive promoter, to provide higher levels of p33ING2 expression. Alternatively, endogenous p33ING2 levels can be examined. The cells can be treated to induce p33ING2 expression. The cells can be immobilized, be in solution, be injected into an animal, or be naturally occurring in a transgenic or non-transgenic animal.

Samples or assays that are treated with a test compound which potentially activates, inhibits, or modulates p33ING2 are compared to control samples that are not treated with the test compound, to examine the extent of modulation. Generally, the compounds to be tested are present in the range from 0.1 nM to 10 mM. Control samples (untreated with activators, inhibitors, or modulators) are assigned relative p33ING2 activity value of 100%. Inhibition of p33ING2 is achieved when the p33ING2 activity value relative to the control is about 90% (e.g., 10% less than the control), optionally 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, or 25-0%. Activation of p33ING2 is achieved when the p33ING2 activity value relative to the control is 110% or more (e.g., at least 10% more than the control), optionally 120%, 130%, 140%, 150% or more, 200-500% or more, 1000-3000% or more.

The effects of the test compounds upon the function of the p33ING2 polypeptides can be measured by examining any of the parameters described above. For example, parameters such as ability to grow on soft agar, contact inhibition and density limitation of growth, growth factor or serum dependence, tumor specific markers levels, invasiveness into Matrigel, apoptosis, G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, tumor growth *in vivo*, transgenic mice and the like, can be measured. Furthermore, the effects of the test compounds on p33ING2 protein or mRNA levels, transcriptional activation or repression of a reporter gene can be measured. In each assay, cells expressing p33ING2 are contacted with a test compound and incubated for a suitable amount of time, e.g., from 0.5 to 48 hours. Then, parameters such as those described above are compared to those produced by control cells untreated with the test compound.



In one embodiment, the effect of test compounds upon the function of p33ING2 can be determined by comparing the level of p33ING2 protein or mRNA in treated samples and control samples. The level of p33ING2 protein is measured using immunoassays such as western blotting, ELISA and the like with a p33ING2 specific antibody. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization, RNase protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

Alternatively, a reporter gene system can be devised using the p33ING2 promoter operably linked to a reporter gene such as luciferase, green fluorescent protein, CAT, or  $\beta$ -gal. After treatment with a potential p33ING2 modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

In another embodiment, the effects of test compounds on p33ING2 activity is performed *in vivo*. In this assay, cultured cells that are expressing a wildtype or mutant p33ING2 (e.g., a null or missense mutation) are injected subcutaneously into an immune compromised mouse such as an athymic mouse, an irradiated mouse, or a SCID mouse. P33ING2 modulators are administered to the mouse, e.g., a chemical ligand library. After a suitable length of time, preferably 4-8 weeks, tumor growth is measured, e.g., by volume or by its two largest dimensions, and compared to the control. Tumors that have statistically significant reduction (using, e.g., Student's T test) are said to have inhibited growth. Alternatively, the extent of tumor neovascularization can also be measured. Immunoassays using endothelial cell specific antibodies are used to stain for vascularization of the tumor and the number of vessels in the tumor. Tumors that have a statistically significant reduction in the number of vessels (using, e.g., Student's T test) are said to have inhibited neovascularization.

Alternatively, transgenic mice with the endogenous p33ING2 gene knocked out can be used in an assay to screen for compounds which modulate the p33ING2 activity. As described in part A, knock-out transgenic mice can be made, in which the endogenous p33ING2 gene is disrupted, e.g., by replacing it with a marker gene. A transgenic mouse that is heterozygous or homozygous for integrated transgenes that have functionally disrupted the endogenous p33ING2 gene can be used as a sensitive *in vivo* screening assay for p33ING2 ligands and modulators of p33ING2 activity.

### C. Modulators

The compounds tested as modulators of p33ING2 can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Alternatively, modulators can be genetically altered versions of p33ING2. For example, an antisense construct of p33ING2 can be used as a modulator.

Typically, test compounds will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such “combinatorial chemical libraries” or “ligand libraries” are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional “lead compounds” or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical “building blocks” such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are



embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the domain, chimeric molecule, p33ING2, or cell or tissue expressing p33ING2 is attached to a solid phase substrate.

In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100-1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-20,000 different compounds is possible using the integrated systems of the invention.

The molecule of interest can be bound to the solid state component, directly or indirectly, via covalent or non covalent linkage, e.g., via a tag. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, *etc.*) Antibodies to molecules with natural binders such as biotin are also widely available and appropriate tag binders; *see*, SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO.

Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair. Thousands of specific antibodies are commercially available and many additional antibodies are described in the literature. For example, in one common configuration, the tag is a first antibody and the tag binder is a second antibody which recognizes the first antibody. In addition to antibody-antigen interactions, receptor-ligand interactions are also appropriate as tag and tag-binder pairs. For example, agonists and antagonists of cell membrane receptors (e.g., cell receptor-ligand interactions such as transferrin, c-kit, viral receptor ligands, cytokine receptors, chemokine receptors, interleukin receptors, immunoglobulin receptors and antibodies, the cadherein family, the integrin family, the selectin family, and the like; *see*,

e.g., Pigott & Power, *The Adhesion Molecule Facts Book I* (1993). Similarly, toxins and venoms, viral epitopes, hormones (e.g., opiates, steroids, etc.), intracellular receptors (e.g. which mediate the effects of various small ligands, including steroids, thyroid hormone, retinoids and vitamin D; peptides), drugs, lectins, sugars, nucleic acids (both linear and cyclic polymer configurations), oligosaccharides, proteins, phospholipids and antibodies can all interact with various cell receptors.

Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature. See, e.g., Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963) (describing solid phase synthesis of, e.g., peptides); Geysen *et al.*, *J. Immun. Meth.* 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, *Tetrahedron* 44:60316040 (1988) (describing synthesis of various peptide sequences on cellulose disks); Fodor *et al.*, *Science*, 251:767-777 (1991); Sheldon *et al.*, *Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al.*, *Nature Medicine* 2(7):753759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by UV radiation, and the like.

### *E. Computer-based assays*

Yet another assay for compounds that modulate p33ING2 activity involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of p33ING2 based on the structural information encoded by the amino acid sequence. The input amino acid sequence interacts directly and actively with a preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, e.g., ligands.

These regions are then used to identify ligands that bind to the protein.

The three-dimensional structural model of the protein is generated by entering p33ING2 amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding a p33ING2 polypeptide into the computer system. The amino acid sequence of the polypeptide or the nucleic acid encoding the polypeptide is selected from the group consisting of SEQ ID NO:1 or SEQ ID NO:2, and conservatively modified versions thereof. The amino acid sequence represents the primary sequence or subsequence of the protein, which encodes the structural information of the protein. At least 10 residues of the amino acid sequence (or a nucleotide sequence encoding 10 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the protein is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art. The three-dimensional structural model of the protein can be saved to a computer readable form and be used for further analysis (e.g., identifying potential ligand binding regions of the protein and screening for mutations, alleles and interspecies homologs of the gene).

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the protein of interest. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy

terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the p33ING2 protein to identify ligands that bind to p33ING2. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein. The results, such as three-dimensional structures for potential ligands and binding affinity of ligands, can also be saved to a computer readable form and can be used for further analysis (e.g., generating a three dimensional model of mutated proteins having an altered binding affinity for a ligand).

Computer systems are also used to screen for mutations, polymorphic variants, alleles and interspecies homologs of p33ING2 genes. Such mutations can be associated with disease states or genetic traits. As described above, high density oligonucleotide arrays (GeneChip™) and related technology can also be used to screen for mutations, polymorphic variants, alleles and interspecies homologs. Once the variants are identified, diagnostic assays can be used to identify patients having such mutated genes. Identification of the mutated p33ING2 genes involves receiving input of a first nucleic acid or amino acid sequence encoding selected from the group consisting of SEQ ID NO:2, or SEQ ID NO:1, and conservatively modified versions thereof. The sequence is entered into the computer system as described above and then saved to a computer readable form. The first nucleic acid or amino acid sequence is then compared to a

second nucleic acid or amino acid sequence that has substantial identity to the first sequence. The second sequence is entered into the computer system in the manner described above. Once the first and second sequences are compared, nucleotide or amino acid differences between the sequences are identified. Such sequences can represent  
5 allelic differences in p33ING2 genes, and mutations associated with disease states and genetic traits.

## VII. Gene Therapy

The present invention provides the nucleic acids of p33ING2 for the  
10 transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well known vectors for the transfection of target cells and organisms as described below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The nucleic acids encoding p33ING2, under the control of a promoter, then expresses a p33ING2 of the present invention,  
15 thereby mitigating the effects of absent, partial inactivation, or abnormal expression of the p33ING2 gene.

Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many  
20 important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, *see* Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology*  
25 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* (Doerfler & Böhm eds., 1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994)).

Delivery of the gene or genetic material into the cell is the first critical step  
30 in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Preferably, the nucleic acids are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal



or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in, e.g., US 5,049,386, US 4,946,787; and US 4,897,355 and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of nucleic acids could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Viral vectors are currently the most efficient and versatile method of gene transfer in target cells and tissues. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-

associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vector that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher *et al.*, *J. Virol.* 66:2731-2739 (1992); Johann *et al.*, *J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

In applications where transient expression of the nucleic acid is preferred, adenoviral based systems are typically used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (see, e.g., West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

In particular, at least six viral vector approaches are currently available for gene transfer in clinical trials, with retroviral vectors by far the most frequently used

system. All of these viral vectors utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

pLASN and MFG-S are examples are retroviral vectors that have been used in clinical trials (Dunbar *et al.*, *Blood* 85:3048-305 (1995); Kohn *et al.*, *Nat. Med.* 1:1017-102 (1995); Malech *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al.*, *Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al.*, *Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al.*, *Hum. Gene Ther.* 1:111-2 (1997).

Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al.*, *Lancet* 351:9117 1702-3 (1998), Kearns *et al.*, *Gene Ther.* 9:748-55 (1996)).

Replication-deficient recombinant adenoviral vectors (Ad) are predominantly used transient expression gene therapy, because they can be produced at high titer and they readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and E3 genes; subsequently the replication defector vector is propagated in human 293 cells that supply deleted gene function in trans. Ad vectors can transduce multiply types of tissues *in vivo*, including nondividing, differentiated cells such as those found in the liver, kidney and muscle system tissues. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Stermann *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al.*, *Infection* 24:1 5-10 (1996); Stermann *et al.*, *Hum. Gene Ther.* 9:7 1083-1089 (1998); Welsh *et al.*, *Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al.*, *Hum. Gene Ther.* 5:597-613 (1997); Topf *et al.*, *Gene Ther.* 5:507-513 (1998); Stermann *et al.*, *Hum. Gene Ther.* 7:1083-1089 (1998).

Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and  $\psi$ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are

usually generated by producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the protein to be expressed. The missing viral functions are supplied in *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. A viral vector is typically modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (e.g., Fab or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells.

Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or

universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

*Ex vivo* cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a nucleic acid (gene or cDNA), and re-infused back into the subject organism (e.g., patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (*see, e.g., Freshney et al., Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

In one embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such as GM-CSF, IFN- $\gamma$  and TNF- $\alpha$  are known (*see Inaba et al., J. Exp. Med.* 176:1693-1702 (1992)).

Stem cells are isolated for transduction and differentiation using known methods. For example, stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells) (*see Inaba et al., J. Exp. Med.* 176:1693-1702 (1992)).

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can be also administered directly to the organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered.

Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells, as described below. The nucleic acids are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route (*see Proc. Natl. Acad. Sci. U.S.A.* 81:6466-6470 (1984); and Samulski *et al., J. Virol.* 63:03822-3828 (1989)).

In particular, at least six viral vector approaches are currently available for gene transfer in clinical trials, with retroviral vectors by far the most frequently used system. All of these viral vectors utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

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#### **VIII. Pharmaceutical Compositions and Administration**

p33ING2 nucleic acid, protein, and modulators of p33ING2 can be administered directly to the patient for inhibition of cancer, tumor, or precancer cells *in vivo*. Administration is by any of the routes normally used for introducing a compound into ultimate contact with the tissue to be treated. The compounds are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such compounds are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

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Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*see, e.g., Remington's Pharmaceutical Sciences*, 17<sup>th</sup> ed. 1985)). For example, if *in vivo* delivery of a biologically active p33ING2 protein is desired, the methods described in Schwarze *et al.* (*see Science* 285:1569-1572 (1999)) can be used.

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The compounds (nucleic acids, proteins, and modulators), alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

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Formulations suitable for parenteral administration, such as, for example, by intravenous, intramuscular, intradermal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous

infusion, orally, topically, intraperitoneally, intravesically or intrathecally. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials. Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

5           The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular compound employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent  
10 of any adverse side-effects that accompany the administration of a particular compound or vector in a particular patient

          In determining the effective amount of the modulator to be administered in the treatment or prophylaxis of cancer, the physician evaluates circulating plasma levels of the modulator, modulator toxicities, progression of the disease, and the production of  
15 anti-modulator antibodies. In general, the dose equivalent of a modulator is from about 1 ng/kg to 10 mg/kg for a typical patient. Administration of compounds is well known to those of skill in the art (*see, e.g., Bansinath et al., Neurochem Res.* 18:1063-1066 (1993); Iwasaki *et al., Jpn. J. Cancer Res.* 88:861-866 (1997); Tabrizi-Rad *et al., Br. J. Pharmacol.* 111:394-396 (1994)).

20           For administration, modulators of the present invention can be administered at a rate determined by the LD-50 of the modulator, and the side-effects of the inhibitor at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

## 25   **IX. Diagnostics and Kits**

          The present invention also provides methods for detection of p33ING2 (either wildtype or mutant). For example, kits are provided that contain p33ING2 specific reagents that specifically hybridize to p33ING2 nucleic acid, such as specific probes and primers, and p33ING2 specific reagents that specifically bind to the protein of  
30 choice, e.g., antibodies. The methods, kits, and the assays described herein can be used for identification of modulators of p33ING2, or for diagnosing patients with mutations in p33ING2.

          Nucleic acid assays for the presence of p33ING2 DNA and RNA in a sample include numerous techniques are known to those skilled in the art. In particular,

p33ING2 specific reagents (e.g., p33ING2-specific primers or nucleic acid probes) can be used to distinguish between samples which contain p33ING2 nucleic acids and samples which contain p33ING1 nucleic acids. Techniques such as Southern analysis, Northern analysis, dot blots, RNase protection, high density oligonucleotide arrays, S1 analysis, amplification techniques such as PCR and LCR, and *in situ* hybridization can be used as assays. In *in situ* hybridization, for example, the target nucleic acid is liberated from its cellular surroundings in such as to be available for hybridization within the cell while preserving the cellular morphology for subsequent interpretation and analysis. The following articles provide an overview of the art of *in situ* hybridization: Singer *et al.*, *Biotechniques* 4:230-250 (1986); Haase *et al.*, *Methods in Virology*, vol. VII, pp. 189-226 (1984); and *Nucleic Acid Hybridization: A Practical Approach* (Hames *et al.*, eds. 1987).

In addition, p33ING2 protein can be detected with the various immunoassay techniques described above, e.g., ELISA, western blotting, and the like. The test sample is typically compared to both a positive control (e.g., a sample expressing recombinant p33ING2) and a negative control. In particular, p33ING2 specific polyclonal and monoclonal antibodies or p33ING1 specific polyclonal and monoclonal antibodies can be used as a diagnostic tool to distinguish between samples which contain p33ING2 antigens and samples which contain p33ING1 antigens. These polyclonal and monoclonal antibodies can also be used to determine the amount of p33ING2 or p33ING1 in samples.

The present invention also provides for kits for screening for modulators of p33ING2. Such kits can be prepared from readily available materials and reagents. For example, such kits can comprise any one or more of the following materials: p33ING2, reaction tubes, and instructions for testing p33ING2 activity. Preferably, the kit contains biologically active p33ING2. Furthermore, the kit may include a label or written instructions for the use of one or more of these reagents and materials in any of the assays described herein. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.



Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

### Example I. Cloning and expression of p33ING2

p33ING1 homologous sequences were found in a random cDNA sequence database consisting of short partial sequences known as expressed sequence tags (ESTs) submitted in GenBank. Using primers designed based on these EST sequences and using RT-PCR and 5'- and 3'- RACE methods, p33ING2 coding region (SEQ ID NO:2) from human placenta cDNA (CLONTECH) was isolated and subcloned into a plasmid .

To obtain a p33ING2 genomic sequence, a human PAC genomic library was screened with the p33ING2 cDNA sequence. Two clones were selected, one of which included the p33ING2 genomic sequence (SEQ ID NO:7; exon1/intron). The genomic structure (exon/intron boundary sequence) was determined by using human PAC genomic clones and "long distance sequence" method. The nucleic acid sequence of SEQ ID NO:10 is exon2/intron of p33ING2 genomic sequence.

Chromosomal localization of p33ING2 was determined using FISH (fluorescent *in situ* hybridization) analysis and human PAC genomic clone including p33ING2 genome as a probe. It was determined that p33ING2 is located at chromosome 4, at 4q35.

One human cancer cell line with a p33ING2 mutation, namely, HCT116, was discovered. As shown in SEQ ID NO:6, it has a missense mutation at amino acid position 153 (Arg to Ser).

### Example II. Cloning and Expression of p33ING1

The p33ING1 mRNA coding and amino acid sequences submitted in GenBank (Accession No. AF044076) had several mistakes. The correct sequence of p33ING1 mRNA coding region was determined by using human placental cDNA and RT-PCR method.

A human PAC genomic library was screened by p33ING1 cDNA sequence. Two clones were picked up which included p33ING1 genomic sequence. The genomic structure (exon/intron boundary sequence) was determined by using the human PAC genomic clones and "long distance sequence" method. The sequence of mRNA coding sequence was also confirmed by the genomic DNA sequence.

### Example III. Antibodies to p33ING2 and p33ING1

Antibodies to p33ING2 and p33ING1 were synthesized using two unique peptides (KMP-1 from p33ING2 (*see, e.g.*, SEQ ID NO:5) and KMP-2 from p33ING1 (*see, e.g.*, SEQ ID NO:9)). These peptides were purified by HPLC; peptide KLH conjugations were made; and rabbits were immunized by them. Antiserum was purified using peptide affinity column and specificity of each polyclonal antibody was analyzed by ELISA.

As shown in Figure 1, by ELISA (enzyme-linked immunosorbent assay) anti-p33ING2 polyclonal antibodies are reactive with recombinant GST-p33ING2 protein or its peptide fragment KMP-1 (SEQ ID NO:5), but are not cross-reactive with recombinant GST-p33ING1 protein or its peptide fragment KMP-2 (SEQ ID NO:9). Anti-p33ING1 polyclonal antibodies are reactive with recombinant GST-p33ING1 protein or its peptide fragment KMP-2, but are not cross-reactive with recombinant GST-p33ING2 protein or its peptide fragment.

As shown in Figure 2, by Western blot analysis, anti-p33ING2 polyclonal antibodies are reactive with recombinant p33ING2 protein, but are not cross-reactive with recombinant p33ING1 protein. Anti-p33ING1 polyclonal antibodies are reactive with recombinant p33ING1 protein, but are not cross-reactive with recombinant p33ING2 protein.

#### Example IV. Inhibition of cell proliferation

The colony formation assay was used to determine if p33ING2 inhibits cell growth of HCT116 cell line (human, hereditary non-polyposis colon cancer cell line, wt p53).

5 Mammalian expression vectors (with CMV promoter, Neomycin resistant) containing p33ING2 in sense orientation (pcDNA3-ING2) and in antisense orientation (pcDNA3-AntiING2) were constructed. HCT116 cell lines were transfected with the expression vectors. The transfected cells were selected by Neomycin. The colony formation assay was used to test the effect of p33ING2 and anti-p33ING2 expression in  
10 HCT116 cell lines. As shown in Figure 3, HCT116 cells transfected with pcDNA3-ING2 formed less colonies compared to HCT116 cells transfected with pcDNA3-AntiING2 or HCT116 cells transfected with pcDNA3 (without any inserts). This result illustrates that p33ING2 inhibits cell growth.

#### Example V. Soft agar assay for identifying compounds that modulate p33ING2

Wildtype or mutant p33ING2 is expressed in host cells to screen compounds that modulate anchorage dependence of host cells expressing p33ING2. This is achieved by using the method disclosed in Garkavtsev *et al.* (1996), *supra*, herein incorporated by reference. Non-tumorigenic immortalized mouse mammary epithelial  
20 cells (NMuMG) are transfected with retrovirus produced from a vector containing p33ING2 in sense or antisense orientation, or a vector lacking insert (control). The soft agar culture is comprised of two layers: an underlay (DMEM, 10% FCS, 0.6% agar) and an overlay (DMEM, 10% FCS, 0.3% agar),  $5 \times 10^4$  cells are plated in soft agar in 10 cm plates are left at 37 °C for 6-7 weeks before being counted. The cells are incubated with a  
25 test compound for a suitable amount of time, e.g., for 0.5 to 48 hours, before counting cells. The amount of cells in the test sample is then compared to control cells untreated with the compound.

#### Example VI. p33ING2 protein induction by DNA damage

30 Calu6 cells were treated with topoisomerase II inhibitor, etoposide (SIGMA, E-1383, 10 µg/ml). Etoposide can induce DNA damage (e.g., double-strand DNA break). Figure 4 shows the Western blot of p33ING1, p33ING2 and beta-actin (as control). The protein analysis indicated that p33ING2 protein expression was induced by

the treatment of Calu6 cells with etoposide. However, p33ING1 protein expression was not induced by etoposide.

Example VII. p33ING1 or p33ING2 can induce G<sub>1</sub> cell cycle arrest

RKO cells were transfected with pcDNA3.1 (control), pcDNA3.1-p33ING1, or pcDNA3.1-p33ING2. Cells were co-transfected with pEGFP-F Amp (a plasmid containing an enhanced green fluorescent protein and an ampicillin transfection marker). The cells were gated by GFP. The GFP-positive cells were considered to be pcDNA3.1, pcDNA3.1-p33ING1, or pcDNA3.1-p33ING2 positive. The propidium iodide signal was used as a measure for DNA content to determine cell cycle profiles on a FACScan flow cytometer (Becton-Dickinson). See Figure 5. The percentages of the cells in each cell cycle phase were calculated by the ModFit program (Becton-Dickinson), and the results are as follows:

pcDNA3.1 G<sub>0</sub>/G<sub>1</sub> (43.1%), G<sub>2</sub>M (32.5%), S-phase (24.4%)

pcDNA3.1-p33ING1 G<sub>0</sub>/G<sub>1</sub> (67.1%), G<sub>2</sub>M (21.7%), S-phase (11.2%)

pcDNA3.1-p33ING2 G<sub>0</sub>/G<sub>1</sub> (71.2%), G<sub>2</sub>M (19.9%), S-phase (8.9%)

These results indicate that p33ING1 or p33ING2 can induce G<sub>1</sub> cell cycle arrest in cells.

Example VIII. p33ING1 and p33ING2 can enhance p21/WAF1, BAX, and IGF BP3 promoter activities in p53 wild type cell line RKO

The p53 transcriptional transactivities (p21/WAF1, BAX, or IGF BP3) were examined with the Dual-Luciferase Reporter Assay System (Promega, E1910). RKO cells were co-transfected with Renilla Luc vector SV 40 (internal control) and p53 responsive reporter vectors, WWP-Luc-p21, PGL3-Luc-BAX, or pUHC13-3-Luc-IGF BP3 BOX B. The cells were also transfected with pcDNA3.1, pcDNA3.1-p33ING1, or pcDNA3.1-p33ING2. The results of the promoter activity according the luciferase assay are as follows:

p21/WAF1 promoter activity (average +/- SD)

pcDNA3.1 (100+/-9.3)

pcDNA3.1-p33ING1 (190.8+/-15.6)

pcDNA3.1-p33ING2 (199.8+/-29.2)

BAX promoter activity (average +/- SD)

pcDNA3.1 (100+/-6.2)

pcDNA3.1-p33ING1 (237.6+/-15.4)

pcDNA3.1-p33ING2 (347.9+/-28.5)

IGF BP3 promoter activity (average +/- SD)

5 pcDNA3.1 (100+/-10.9)

pcDNA3.1-p33ING1 (181.8+/-20.6)

pcDNA3.1-p33ING2 (205.0+/-13.1)

The above results indicate that p33ING1 and p33ING2 enhanced p21/WAF1, BAX, and  
10 IGF BP3 promoter activities in p53 wild type cell line RKO.

Example IX. p33ING1 and p33ING2 induces apoptosis

RKO cells were transfected with pcDNA3.1 (control), pcDNA3.1-  
p33ING1, or pcDNA3.1-p33ING2 expression vector. Cells were co-transfected with  
15 pEGFP-F Amp (transfection marker). The cells were fixed 24 hours after transfection.  
The GFP-positive cells were considered to be pcDNA3.1, pcDNA3.1-p33ING1, or  
pcDNA3.1-p33ING2 positive. Apoptotic change was determined by DAPI staining and  
TUNEL assay using fluorescent microscope. For TUNEL assay, the following kit and  
materials were used: Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene  
20 Research Products, Cat.# QIA39) + Tetramethyl-rhodamine-5-dUTP (Roche, Cat. # 1534  
378).

The assay results are as follows.

% apoptotic cells / transfected cells (GFP-positive cells)

25 pcDNA3.1 (control): 15.3 +/- 1.3 (average +/- SD)

pcDNA3.1-p33ING1: 40.3 +/- 3.0

pcDNA3.1-p33ING2: 39.3 +/- 1.7

The above results indicate that expression or overexpression of p33ING1 or p33ING2  
induced apoptosis in RKO cells at a higher frequency compared to the control.